Supplementary Materials

Molecular Biology of the Cell

Hansen *et al*.

Supplementary Material

MotiQ: an open-source toolbox to quantify the cell motility and morphology of microglia

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Supplementary Note 1: *MotiQ cropper*

MotiQ cropper is a tool for defining and extracting regions of interest (ROIs) from image stacks and time series and is used to generate single-cell images of microglia. Any file format that is compatible with ImageJ can be processed. *MotiQ cropper* displays and guides through z slices and time frames for ROI selection and automatically clears any signal outside of a ROI. After complete processing of a given image stack, *MotiQ cropper* automatically saves the processed stack in the TIFF format in the same directory as the original image file. In addition, all information pertaining to the ROI is automatically saved as a ZIP archive and a metadata file in the TXT format. If the <Post processing, crop image to selected region> is activated in the initial dialog of *MotiQ cropper*, the processed image stack is automatically cropped to its minimum required size (in x, y, z) in order to save storage space.

Supplementary Note 2: *MotiQ thresholder*

MotiQ thresholder is a tool for automated image segmentation of microscopic images obtained with *MotiQ cropper*. In addition, any other 8-bit, 16-bit, or 32-bit image can be processed with *MotiQ thresholder*. If images are processed that are not obtained by MotiQ cropper, <Use alternate reference image> may be unselected in the settings dialog.

We introduced several preprocessing steps into *MotiQ thresholder* that we found useful when analyzing different microglia imaging data sets. The aim of *MotiQ thresholder* is to standardize and automatize image preprocessing and segmentation. MotiQ thresholder settings may need to be initially optimized to the user's data set. Later, the same *MotiQ thresholder* setting shall be applied to all images of a data set.

Images are processed following a three-step approach.

First, a reference image of a given input image is generated and pre-processed to optimize its histogram for threshold calculation. This is achieved by (A) down-scaling image resolution, (B) rescaling the intensity histogram for better coverage of the whole bit range, (C) including

multiple time frames or z stacks in the histogram, and/or (D) converging the relationship of fore- and background pixel numbers towards equilibrium. These methods can be selected together or individually in the setting options of *MotiQ thresholder* (Supplementary Table 1). The different options A-D to preprocess the image were included into *MotiQ thresholder* to support accurate determination of an intensity threshold for image segmentation.

(A) Down-scaling image resolution can improve threshold calculation as it suppresses camera / detector noise in the image. During down-scaling in *MotiQ thresholder*, neighboring pixels are binned into one pixel. Thereby the stochastic noise from the detector is averaged and foreground and background may become more distinguished in the image's intensity histogram. In turn, the threshold calculation may include also dim parts of the cell that otherwise would be so similar to noise pixels that they will not be included in the foreground (Supplementary Figure 1). At the same time, more noise particles may be detected as foreground since the threshold will be calculated too low to exclude all background noise. However, these can later be removed by MotiQ 2D or 3D analyzer through the particle-size filter.

(B) Rescaling the intensity histogram to an optimal coverage of the intensity range allows to normalize the overall intensity level for each image. To rescale the intensity histogram, *MotiQ thresholder* determines the maximum and the minimum intensity in the single-cell image and in the original image, at the region from which the single-cell image was derived. Next, the intensities in the image are linearly rescaled so that the minimum intensity value in the image corresponds to 0 and the maximum intensity value corresponds to 255. Thereafter, the image is converted to an 8-bit image. This rescale and conversion function allows to normalize the intensity values of fore and background so that all images within a data set feature a similar intensity range. Thereby, the same intensity threshold method may perform more comparable for all cells within a data set.

(C) Including multiple time frames / z stacks into threshold calculations can provide a less noisy intensity histogram, since more voxels in total are included. Note: If the user analyzes a time-lapse recording, it is possible to segment different time spans (e.g. the first 5 frames, frames 6-10, and frames 11-15) separately by using the <Threshold only distinct time series> option in MotiQ thresholder and running the image multiple times through MotiQ thresholder, one time for each selection of time frames. This also allows to adapt the segmentation strategy to overall changes in intensity level due to photobleaching.

(D) Converging the relationship of fore- and background pixel numbers towards equilibrium refers to *MotiQ thresholder*'s function to determine the intensity threshold in the intensity histogram obtained from a maximum intensity projection of the z-stack instead of in the intensity histogram obtained from the whole z-stack (Supplementary Figure 2a). Since microglia with their fine processes do not cover a high number of pixels per individual confocal slice, distinguishing the peak from foreground and background in an intensity histogram of individual confocal slices or the whole stack can be difficult; Especially when the intensity difference between background noise and microglial signals is small the intensity peak from the large number of background pixels may hide the peak from the small number of foreground pixels. Furthermore, especially in large z-stacks spanning a wide depth of tissue, the intensity level of fore and background may change from slice to slice. Thus, from a stack histogram the background may be inaccurately estimated too low since it may vary across tissue depth (Supplementary Figure 2a,b). Determining the intensity threshold in the maximum projection will detect the highest background signals as background and at the same time contain a larger percentage of microglia pixels compared to a whole stack histogram. This can improve the threshold estimate (Supplementary Figure 2a,b).

Second, based on the processed reference image, an intensity threshold is calculated using one of the currently available ImageJ intensity thresholding algorithms (e.g., Default, IJ_IsoData, Huang, Intermodes, IsoData, Li, MaxEntropy, Mean, MinError, Minimum, Moments, Otsu, Percentile, RenyiEntropy, Shanbhag, Triangle, Yen). The algorithm is pre-defined by the user and should be selected based on best separation of fore and background in the user's images.

Third, the calculated intensity threshold is applied to the original un-processed input image. Depending on the selected settings, the output image is either a background-depleted image with fully preserved intensity information or a binary image, in which all pixels above the threshold are set to maximum.

There are two options for generating reference images. For images not obtained by *MotiQ cropper,* a copy of the input image is automatically generated and used as a reference image. For images obtained by *MotiQ cropper*, an alternate image (i.e., the copy of the original image before processing with *MotiQ cropper*) is generated and used as a reference image. This ensures that cleared areas and limited background information due to processing with *MotiQ cropper* do not hamper threshold calculation.

Furthermore, there are different options for processing time series. For example, in time series with significant photo bleaching, time steps can be processed individually. Alternatively, sets of time steps of a given time series can be selected and processed together. This facilitates processing of time series, in which the fluorescence intensity changes at distinct time points e.g., due to compound addition. In both cases, *MotiQ thresholder* automatically splits, individually processes and remerges time steps of time series.

MotiQ thresholder also offers four options for optimized threshold calculation in image stacks (z stacks and/or time frames; Supplemental Table 1):

1) When option <apply average threshold of independent stack-image thresholds> is selected, a threshold for every stack image is individually calculated, averaged and applied to the

individual stack images. This option should only be selected, if size and fluorescence intensity levels of cell and background do not largely vary between stack images.

2) When option <apply threshold determined in the stack histogram> is selected, a threshold is calculated using a histogram with all intensity values of the image stack and applied to the stack images. Again, this option should only be selected, if fluorescence intensity levels of cell and background do not largely vary between stack images. However, threshold calculation in this option is independent of the distribution of the cell in z slices and may thus contain "empty" z slices with only background and no cell.

3) When option <threshold every stack image independently> is selected, a threshold is calculated and applied in each stack image. This option is recommended for image stacks, in which the intensity levels of cell and/or background vary between stack images e.g., due to photo bleaching or imaging in deep tissue. However, this option should only be selected if the cell spans through all z slices/stack images. Alternatively, an intensity normalization approach can be used prior to *MotiQ thresholder* processing e.g., by using the option *Normalize* in the ImageJ function *Enhance contrast* or the ImageJ plugin *CorrectBleach*. Afterwards, the image stack can be processed using options 1, 2, or 3 of *MotiQ thresholder*.

4) When option <apply threshold determined in a maximum-intensity-projection> is selected, a threshold is calculated using the maximum-intensity projection of the image stack and applied to the stack images. This option should only be selected if the fluorescence intensity level is similar in all stack images.

MotiQ thresholder also contains a local threshold calculation method. If chosen, *MotiQ thresholder* calculates an individual threshold for each pixel in the image, based on the pixel intensity values within a user-defined radius around the specific pixel.

To enhance user-friendliness, *MotiQ thresholder* contains a multi-task management option to process large data sets.

All *MotiQ thresholder* settings have been extensively tested and a list with optimized settings for specific imaging data is provided in Supplementary Table 1.

Supplementary Note 3: *MotiQ 2D* and *3D analyzer*

MotiQ 2D and *3D analyzer* are automated cell analysis tools that process background-depleted or binary images obtained by *MotiQ thresholder*. *MotiQ 2D analyzer* processes 2D images or time series, including 2D projections of z stacks. *MotiQ 3D analyzer* processes z stacks and time series of z stacks. Both tools use a Flood-Filler algorithm to detect individual objects that are connected in two (*MotiQ 2D analyzer*) or three spatial dimensions (*MotiQ 3D analyzer*) and/or over time (4D). Any voxel that has an intensity value above zero is considered foreground and included in the connection process. Objects below a user-defined voxel size are considered noise and removed from the image. For quantification, the voxels of all remaining objects are considered as one object (for single-cell images generated by *MotiQ cropper*), or are analyzed separately as individual objects (for images not processed with *MotiQ cropper* and containing multiple cells). Objects in consecutive time frames are connected to one object when they overlap at least in one voxel position.

All detected objects/cells are automatically analyzed, including their skeleton and convex hull, resulting in 62 object parameters (Supplementary Tables 2 - 4). Morphological (Supplementary Table 2) and signaling parameters (Supplementary Table 4) are determined in each time step. Motility parameters (Supplementary Table 3) are determined by comparing consecutive time frames.

To reconstruct and analyze the cell skeleton, the ImageJ plugins *Skeletonize3D* (source code: https://github.com/fiji/Skeletonize3D/) and *AnalyzeSkeleton* (source code: https://github.com/fiji/AnalyzeSkeleton) by Ignacio Arganda-Carreras were integrated in *MotiQ 2D and 3D analyzer*.

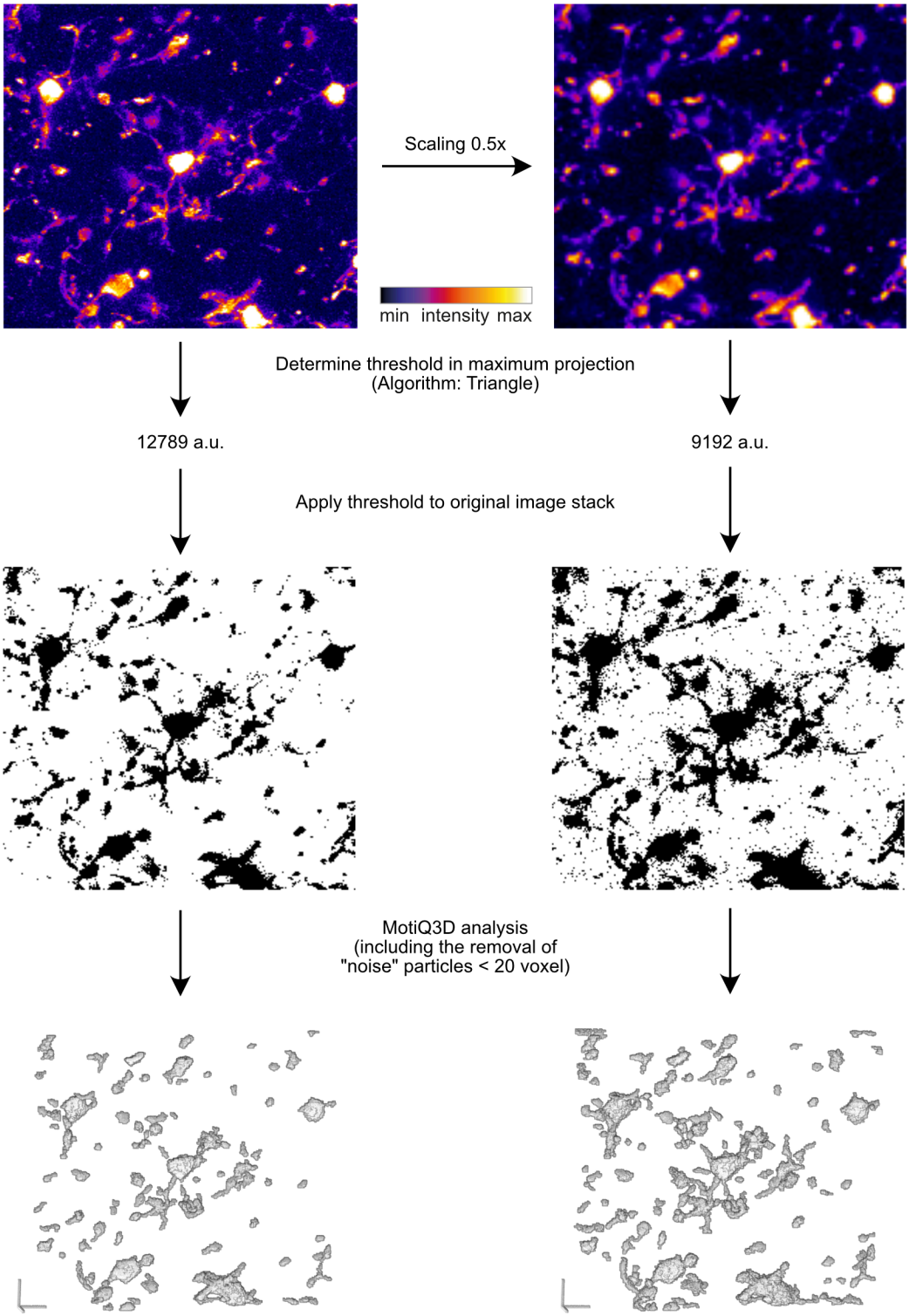
In some cases, especially in images with high noise level, multiple skeletons are erroneously detected for a single cell. To adjust for this problem, skeleton parameters are both analyzed for all detected cell skeletons and for the largest cell skeleton (Supplementary Table 2). When only one skeleton is detected, the results for all skeletons and the largest skeleton are identical. Thus,

comparing skeleton analysis results aids in uncovering errors in automated skeleton detection and/or user-dependent cropping. Furthermore, comparing these data aids in uncovering false automated cell separation by *MotiQ analyzer*, when processing images with multiple cells.

The convex hull of a cell is generated and analyzed using different algorithms. In *MotiQ 2D analyzer* all pixels of a given cell are collected in a polygon, which is transferred into a ROI and transformed into a convex hull polygon using the ImageJ function *ROI.getConvexHull()*. The convex hull polygon is then written back to an image with the same calibration as the input image. The resulting object is detected by a Flood-Filler algorithm and analyzed (Supplementary Table 2). In *MotiQ 3D analyzer* the convex hull of a cell is determined using a custom-written algorithm. Here, the surface pixels of a given object are transformed into a polygon in each z slice. The polygon is then transformed into a convex hull polygon using the ImageJ function *ROI.getConvexHull()*. Every point of the resulting convex hull polygon is connected to corresponding points of the convex hull polygons in other z slices by virtual lines. Intersection points of the virtual lines are added to the polygon of the respective slice image and the polygon in each slice image is again transformed into a convex hull polygon. These convex hull polygons are then written back into an image stack with the same resolution as the original image stack. The resulting 3D object is detected by a Flood-Filler algorithm and analyzed (Supplementary Table 2).

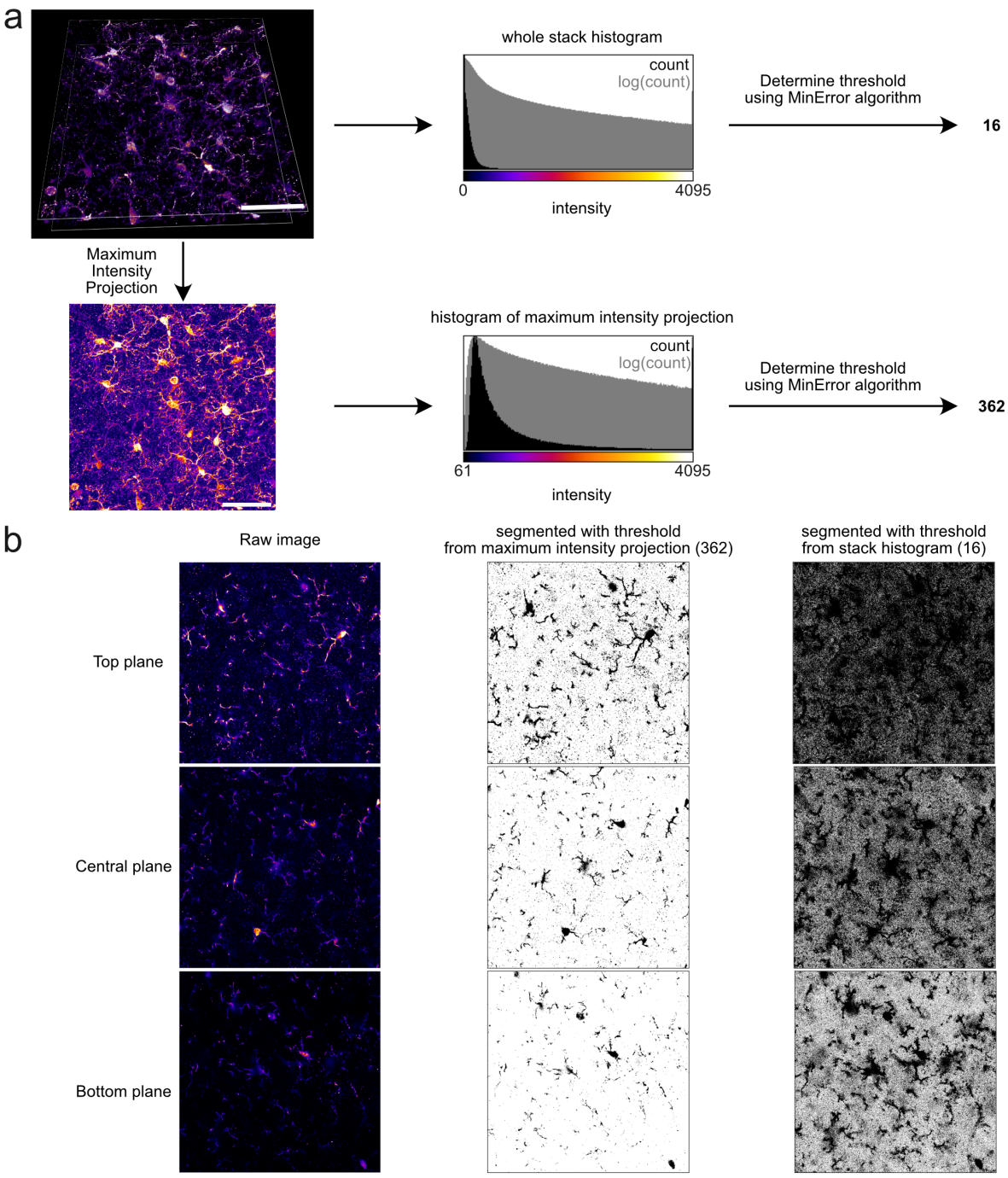
At the end of the analysis process, *MotiQ 2D* or *3D analyzer* generate three images, visualizing (1) the area (2D) or volume (3D) of the object, (2) its convex hull, and (3) its skeletal structure (Figures 1-2; Supplementary Figures 1-2; Supplementary Videos 1-5). Furthermore, *MotiQ 3D analyzer* generates 3D visualizations of these three cell characteristics. This function was integrated in *MotiQ* by using a modified code of the ImageJ plugin *Volume Viewer* by Kai Uwe Barthel (original source code: https://github.com/fiji/Volume_Viewer). Furthermore, TXT files, containing all results and analysis settings are saved in the same directory as the original

image together with the output images. *MotiQ 2D* and *3D analyzer* both contain multi-task management options.



Supplementary Figure 1: Down-scaling noisy images for threshold calculation can improve segmentation.

Maximum projections are shown for better illustration (although a 3D stack is processed). For details see Supplementary Note 2.



Supplementary Figure 2: Determining the intensity threshold in a maximum projection can improve segmentation

For details see Supplementary Note 2. Scale bars: 50 µm.

Supplementary Table 1: *MotiQ thresholder* - optimized settings

Data / Experiment	Restrict calculation to position of cropped image	Convert image to 8 bit[i]	Threshold every time step seperately	Stack handling	Scaling factor	Threshold calculation algorithm	Fill holes	Keep fluorescence intensities above threshold
3D two-photon microscopy time series (*in vivo* or *ex vivo*)	yes	no	yes	maximum intensity projection	0.5	MinError	no	yes
2D MIP[ii] two-photon microscopy time series (*in vivo* or *ex vivo*)			no	stack histogram				
3D confocal microscopy images (e.g., immunofluorescent-labeled cerebral tissue section)		yes	no	maximum intensity projection		MinError (Li or Huang, if image has high noise level)		
2D MIP[i] confocal microscopy images (e.g., immunofluorescent-labeled retinal whole mounts)		no		no stack image				
2D epifluorescence microscopy time series (e.g., fluorescent 2D-cultured microglial cells)		no	no	each z slice indepen-dently		MinError	yes or no	
3D spinning-disk confocal microscopy time series (e.g., fluorescent 3D-cultured microglial cells)				stack histogram		Huang	yes	
3D confocal microscopy images for automatic cell seperation	no	no	no	maximum intensity projection		Li[iii]	no	

[i] Prior to 8-bit conversion, intensity values are rescaled, normalizing the overall intensity level (see Supplementary Note 2).
[ii] MIP: maximum-intensity projection
[iii] To better separate adjacent microglial cell processes, a harsher threshold algorithm was selected.

Supplementary Table 2: *MotiQ analyzer* - morphology parameters

Parameter	Description
Center	Binary average position of all cell voxels[iv]
Center of mass	Intensity-weighted average position of all cell voxels
Area (2D) or volume (3D)	Cell size 2D: $\#voxels \cdot voxelWidth \cdot voxelHeight$ 3D: $\#voxels \cdot voxelWidth \cdot voxelHeight \cdot voxelDepth$
Contour (2D) or surface (3D)	Cell surface 2D: $\sum_{i=0}^{\#\,voxels} contour_{voxel\ i}$ $contour_{voxel\ i}$ is defined as the contour of the voxel i, at which (a) neighbored voxel(s) is/are of zero intensity 3D: $\sum_{i=0}^{\#\,voxels} surface_{voxel\ i}$ $surface_{voxel\ i}$ is defined as the border surfaces of the voxel i, at which (a) neighbored voxel(s) is/are of zero intensity
2D or 3D ramification index	Degree of ramification (complexity of cell shape) 2D: $\frac{contour}{2\sqrt{\pi \cdot area}}$ 3D: $\frac{surface}{4\pi \cdot ((3 \cdot \mathrm{volume})/(4\pi))^{2/3}}$
# found skeletons[v]	Number of separate skeletons (= process trees) detected[v]
# branches (largest skeleton[vi])	Number of continuous stretches (without crossings) in the largest detected skeleton
# junctions (largest skeleton[vi])	Number of crossings (where ≥ 3 branches meet) in the largest detected skeleton
# tips (largest skeleton[vi])	Number of points in the largest detected skeleton that have exactly 1 neighbored point
# triple points (largest skeleton[vi])	Number of crossings in the largest detected skeleton where 3 branches meet
# quadruple points (largest skeleton[vi])	Number of crossings in the largest detected skeleton where 4 branches meet
# junction voxels (largest skeleton[vi])	Number of voxels in the largest skeleton that have > 2 neighboring voxels
# slab voxels (largest skeleton[vi])	Number of voxels in the largest skeleton that have exactly 2 neighboring voxels
Tree length (largest skeleton[vi])	Length sum of all branches in the largest skeleton
Average branch length (largest skeleton[vi])	Average length of all cell branches in the largest skeleton
Maximum branch length (largest skeleton[vi])	Maximum length of single branches in the largest skeleton
Shortest path (largest skeleton[vi])	Largest shortest path in the largest skeleton (determined using the Floyd - Warshall algorithm)
# branches (all skeletons[vi])	Corrected number of branches of all found skeletons 2D/3D: $\#branches_{skeleton\ 1} + \sum_{n=2}^{\#found\ skeletons} (\#branches_n - 1)$
# junctions	Sum of the numbers of junctions in all found skeletons

[iv] A voxel in 3D analysis represents a pixel in 2D analysis

[v] MotiQ contains the ImageJ-plugins *Skeletonize3D* and *AnalyzeSkeleton* by Arganda-Carreras et al. (2010) to generate and analyze the cell skeleton. In some cases, multiple cell skeletons are erroneously detected for one cell.

[vi] As sometimes multiple cell skeletons are detected for one cell, *MotiQ* provides results for the largest cell skeleton and for a mathematical combination of all cell sekeletons. If only one cell skeleton is detected, both sets of parameters show the same result.

Parameter	Description
(all skeletons[vi])	
# tips (all skeletons[vi])	Corrected number of tips of all found skeletons 2D/3D: $\#tips_{skeleton\ 1} + \sum_{n=2}^{\#found\ skeletons}(\#tips_n - 1)$
# triple points (all skeletons[vi])	Sum of the numbers of triple points in all found skeletons
# quadruple points (all skeletons[vi])	Sum of the numbers of quadruple points in all found skeletons
# junction voxels (all skeletons[vi])	Sum of the numbers of junction voxels in all found skeletons
# slab voxels (all skeletons[vi])	Sum of the numbers of slab voxels in all found skeletons
Tree length (all skeletons[vi])	Sum of branch length in all skeletons
Average branch length (all skeletons[vi])	Average branch length in all skeletons
Maximum branch length (all skeletons[vi])	Maximum branch length in all skeletons
Shortest path (all skeletons[vi])	Sum of the largest shortest paths of all found skeletons
Spanned area (2D) or spanned volume (3D)	2D: area of the convex hull 3D: volume of the convex hull Similarly determined as area (2D) or volume (3D), see above. For details on convex hull generation and analysis, see Supplementary Note 3.
Spanned contour (2D) or spanned surface (3D)	2D: contour of the cell's convex hull 3D: surface area of the cell's convex hull Similarly determined as contour (2D) or surface (3D), see above. For details on convex hull generation and analysis, see Supplementary Note 3.
Spanned area center (2D) or spanned volume center (3D)	Center of the cell's convex hull
Binary polarity vector	2D: $spanned\ area\ center - center$ 3D: $spanned\ volume\ center - center$
Binary polarity vector length	Length of the binary polarity vector 2D/3D: $\lvert binary\ polarity\ vector \rvert$
Binary polarity index	Normalized form of the binary polarity vector length 2D: $\frac{\lvert binary\ polarity\ vector \rvert}{2 \cdot \sqrt{spanned\ area\ /\ \pi}}$ 3D: $\frac{\lvert binary\ polarity\ vector \rvert}{2 \cdot \sqrt[3]{3 \cdot spanned\ volume/(4\pi)}}$
Polarity vector	2D: $spanned\ area\ center - center\ of\ mass$ 3D: $spanned\ volume\ center - center\ of\ mass$ Takes into account positions of high intensity regions (e.g. cell soma) within the cell.
Polarity vector length	Length of the polarity vector 2D/3D: $\lvert polarity\ vector \rvert$.
Polarity index	Normalized form of the polarity vector length 2D: $\frac{\lvert polarity\ vector \rvert}{2 \cdot \sqrt{spanned\ area\ /\ \pi}}$ 3D: $\frac{\lvert polarity\ vector \rvert}{2 \cdot \sqrt[3]{3 \cdot spanned\ volume/(4\pi)}}$

Supplementary Table 3: *MotiQ analyzer*: motility parameters

Parameter	Description
Extended area[vii] (2D) or volume[vii] (3D)	Extended area[vii] (2D) or volume[vii] (3D) in comparison to the previous time step (normalized to 1/time unit)
Retracted area[vii] (2D) or volume[vii] (3D)	Retracted area[vii] (2D) or volume[vii] (3D) in comparison to the previous time step (normalized to 1/time unit)
Shape dynamics	Overall cellular shape alteration, compared to the previous time step (normalized to 1/time unit) 2D: $extended\ area + retracted\ area$ 3D: $extended\ volume + retracted\ volume$
Area[vii] (2D) or volume[vii] (3D) alteration	Change in size compared to the previous time step (normalized to 1/time unit)
2D or 3D ramification index alteration	Change in ramification (shape complexity) compared to the previous time step (normalized to 1/time unit)
# extensions	Number of newly formed connected areas (2D) or volumes (3D), compared to the previous time step (normalized to 1/time unit)
# retractions	Number of diminished connected areas (2D) or volumes (3D), compared to the previous time step (normalized to 1/time unit)
# extensions (> 1 voxel[viii])	Number of newly formed connected areas (2D) or volumes (3D) larger than 1 voxel[viii], compared to the previous time step (normalized to 1/time unit); less sensitive to noise
# retractions (> 1 voxel[viii])	Number of diminished connected areas (2D) or volumes (3D) larger than 1 voxel[viii], compared to the previous time step (normalized to 1/time unit); less sensitive to noise
Scanned area[vii] (2D) or volume[vii] (3D)	Area[vii] (2D) or volume[vii] (3D) that is scanned over a defined time period Determined by projecting all images of a user-defined time period into one image, where the size of the projected particle is calculated
Static area[vii] (2D) or volume[vii] (3D)	Indicates the volume of the cell, which is not moving (static) over a defined time period Determined by the area[vii] (2D) or volume[vii] (3D) in the image, where the cell has been continuously present over a defined time period
Scanning activity	Surveillance activity of the cell 2D: $(scanned\ area - static\ area)/scanned\ area$ 3D: $(scanned\ volume - static\ volume)/scanned\ volume$
Scanned area[vii] by the spanned area (2D) or scanned volume[vii] by the spanned volume (3D)	Area[vii] (2D) or volume[vii] (3D) that is scanned by the cell's convex hull during a defined time period
Static spanned area[vii] (2D) or volume[vii] (3D)	Indicates the cell's convex hull volume that is not moving over a defined time period
Spanned area activity (2D) or spanned volume activity (3D)	Activity of the cell in changing its convex hull 2D: $(scanned\ convex\ hull\ area - static\ area)/scanned\ area$ 3D: $(scanned\ volume - static\ volume)/scanned\ volume$
Binary moving vector length	Displacement of the cell center compared to the previous time step; Indicator for directed movement / migration
Moving vector length	Displacement of the center of mass compared to the previous time step; Indicator for directed movement / migration, especially the movement of high intensity regions within the cell

[vii] Area (2D) and volume (3D) are determined analogous to the parameters cell area (2D) and volume (3D) (Supplementary Table 2).
[viii] a voxel in 3D analysis represents a pixel in 2D analysis

Parameter	Description
Binary accumulated distance	Track length of the center over a defined time period
Binary Euclidean distance	Euclidean displacement of the center comparing the first to the last time step of a defined time period
Binary directionality index	Directionality of the center displacement 2D/3D: $\frac{binary\ Eucledian\ distance}{binary\ accumulated\ distance}$
Accumulated distance	Track length of the center of mass over a defined time period
Euclidean distance	Euclidean displacement of the center of mass comparing the first to the last time step of a defined time period
Directionality index	Directionality of the center of mass displacement 2D/3D: $\frac{Eucledian\ distance}{accumulated\ distance}$

Supplementary Table 4: *MotiQ analyzer:* signaling parameters

Parameter	Description/calculation
Average intensity	Average intensity of all cell voxels[ix]
Minimum intensity	Minimum intensity of all cell voxels Can be used to normalize the average or maximum intensity, e.g., when fluorescence signals of interest are exclusively located in distinct cellular compartments
Maximum intensity	Maximum intensity of all cell voxels
Standard deviation of intensity	Standard deviation of the intensities of all voxels A low standard deviation indicates a homogeneous distribution of the fluorescence intensity. A high standard deviation indicates heterogeneous signal localization in the cell or in selected cellular compartments.
Fluorescence intensity levels and distribution also affect several morphological and dynamic parameters such as the center of mass, polarity parameters, the moving vector length, or the directionality index (Supplementary Tables 2 and 3).	

[ix] a voxel in 3D analysis represents a pixel in 2D analysis

Supplementary Video 1: 2D analysis of *in vivo* two-photon microscopy data

2D analysis of a murine cortical microglial cell imaged by *in vivo* two-photon microscopy in CX3CR1$^{EGFP/wt}$ mice. Left: Time series of maximum-intensity projections before *MotiQ* analysis. Middle: Output images of the detected cell and its convex hull (i.e. spanned area, white line). Right: Output images of the detected cell skeleton (orange: branches; blue: tips; magenta: junctions). Time format, mm:ss. Scale bar, 20 µm.

Supplementary Video 2: 3D analysis of *in vivo* two-photon microscopy data

3D analysis of a murine microglial cell imaged by *in vivo* two-photon microscopy in CX3CR1$^{EGFP/wt}$ mice. Left: Time series of maximum-intensity projections before *MotiQ* analysis. Middle left: Output images of the 3D-reconstructed cell. Middle right: Output images of the convex hull (i.e. spanned volume). Right: Output images of the cell skeleton (orange: branches; blue: tips; magenta: junctions). Time format, mm:ss. Scale bar, 20 µm.

Supplementary Video 3: Analysis of 2D-cultured primary microglia

Analysis of a primary CX3CR1$^{EGFP/wt}$ microglial cell in a 2D culture system, imaged by live-cell epifluorescence microscopy. Images were recorded before and after addition of ATP (100 µM). Left: Time series of epifluorescence microscopy images before *MotiQ* analysis. Right: Output image of the detected cell and its convex hull (i.e. spanned area, white line). Time format, mm:ss. Scale bar, 20 µm.

Supplementary Video 4: Analysis of 3D-cultured primary microglia

3D analysis of a primary Lifeact–GFP$^{+/-}$ microglial cell in a 3D culture system obtained by live-cell spinning-disc confocal microscopy. Images were recorded before and after ATP perfusion (100 µM). Left: Time series of maximum-intensity projections before *MotiQ* analysis. Middle left: Output images of the 3D-reconstructed cell. Middle right: Output images of the

convex hull (i.e. spanned volume). Right: Output images of the cell skeleton (orange: branches; blue: tips; magenta: junctions). Time format, mm:ss. Scale bar, 20 µm.

Supplementary Video 5: Analysis of cellular calcium signals and motility

Analysis of a 2D-cultured bone marrow-derived macrophage expressing the genetically encoded calcium indicator GCaMP3 obtained by live-cell epifluorescence microscopy. Images were recorded before and during perfusion with ATP (5 mM). Time series of epifluorescence microscopy images before *MotiQ* analysis. Right: Output images of the detected cell, illustrating changes in fluorescence intensity and the cell's convex hull (i.e. spanned area, white line). Time format, mm:ss. Scale bar, 20 µm.